In Vitro Study of Ambient Air Pollutants on Endothelial Cells

Honors Research Thesis

Presented in Partial Fulfillment of the Requirements for Graduation

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ABSTRACT

Ambient fine particulate matter ($PM_{2.5}$, diameter less than 2.5 μm) is a major component of air pollutants. Because of the small sizes of particulate categorized as $PM_{2.5}$, it can easily be inhaled deeply into the lungs and deposit in the alveoli. These small particles may cross the air-liquid interface, enter the blood stream, and spread to the rest of the body. Studies over the past few decades have linked $PM_{2.5}$ to different health consequences including cardiovascular diseases by causing local or systemic damage. Moreover, it has been well recognized that toxicity of $PM_{2.5}$ depends, in part, on the specific chemical composition of the particles, such as the metals Ni, Mn, Al, Cu, which are often implicated as causative agents of disease. Currently, it is known that $PM_{2.5}$ can exacerbate inflammation of endothelial tissues, leading to health defects including cardiovascular disease, insulin resistance, and type II diabetes.

Among the $PM_{2.5}$ and Ni exposed tissues, we are specifically interested in the endothelium. Local macrophages could be activated by this air contaminant combination. In turn, the activated macrophages will recruit more circulating macrophages to this specific region and induce local inflammation. This is especially important because local inflammation is a key player in suppressing eNOS which supports vasal activity.

Hence, we tested the hypotheses that the exposure to $PM_{2.5}$ in combination with a transition metal such as Ni would result in inflammatory responses as well as a reduction of vasodilatation through the endothelial nitric oxidase synthase (eNOS) related pathways. The chemotaxis of murine macrophage-like cell line RAW 264.7 was activated by $PM_{2.5}$ and further induced with the addition of Ni particles. The activated M1 like macrophages in turn induced more macrophage accumulation. In terms of endothelial function, the suppressed eNOS expression by activated macrophages and local inflammation implies the loss of vassal activity.

BACKGROUND

Over the last several decades, studies have shown that air pollution has severe and detrimental effects on health. This includes both acute and chronic consequences to the lungs, heart, and other organs.[i] Air pollution involves suspended particulate and dissolved gaseous contaminants composed of many different toxic components. In particular, ambient fine particulate matter ($PM_{2.5}$) including metals (i.e. Cd, Cr, Cu, Fe, Mn, Ni, Pb, V, and Zn) have been identified as a major components of air contaminants.[ii] Due to the small sizes, $PM_{2.5}$ can easily be inhaled into the respiratory system and enter the rest of body via the circulatory system. $PM_{2.5}$ can arise from a variety of sources. Urbanization has caused an increase of $PM_{2.5}$ from exhaust from combustion enginges and processes due to increased car usage, manufacturing factories, and coal powered power plants. Many individuals are also subject to the hazards of extended exposure to $PM_{2.5}$ due to their occupation such as individuals working in construction or near areas of heavy pollution.[iii] Thus, air pollutants, such as particulate matter, are affecting a wide range of individuals, and it is important to study the mechanisms by which the body responds to these environmental stimuli. There has been an increasing association between $PM_{2.5}$ exposure and increased cardiovascular health risks[iv]; however, the involved mechanisms are still unclear. Recently, it has been reported that the body's response to air contaminants and especially $PM_{2.5}$ are very similar to that of obesity.[v]

Finally, the function of the endothelial cells will also be altered by the $PM_{2.5}$ and nickel exposure. Endothelium derived nitric oxide synthase (eNOS) is important for proper cardiovascular function.[vi] However, eNOS activity is vulnerable to an inflammatory status. As shown by previous studies[vii], $PM_{2.5}$ in combination with Ni could be a strong inducer of the M1 pro inflammatory macrophage. Thus, in the presence of these pollutants, the activated

macrophages can induce the inflammatory status of endothelial cells which will impair cardiovascular function via suppression of eNOS expression. This will lower vasodilation which often leads to atherosclerosis.[viii]

METHODS

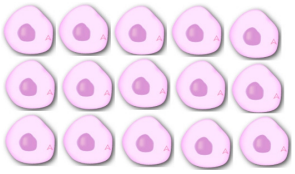
Cell Culture and Pollutant Exposure

Murine macrophage-like cell RAW264.7 were cultured at 37°C in DMEM (Dulbecco's Modified Eagle Medium) medium with 10% FBS (fetal bovine serum), and 1% of penicillin and streptomycin. The RAW cells were cultured in six well plates until 100% confluence and then exposed to varying concentrations of particulate matter and nickel. The exposure was a period of 16 hours. There were three sets of treatment. The first treatment of the RAW cells contained only $PM_{2.5}$. One well acted as the control with no $PM_{2.5}$, and the other five had the following concentrations of $PM_{2.5}$: 6.25 ug/mL, 12.5 ug/mL, 25 ug/mL, 50 ug/mL, and 100 ug/mL. The next trial was run with only nickel. Again, one well acted as the control with no nickel, and the other five had the following nickel concentrations: 10^{-10} M, 10^{-9}M, 10^{-8} M, 10^{-7} M, and 10^{-6} M. Finally, the third trial had a mixture of $PM_{2.5}$ and nickel. The first well again acted as the control and the other five had $PM_{2.5}$ concentrations of 25 ug/mL with the varying concentrations of nickel of: 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, and $2x10^{-4}$ M. The varying concentrations of nickel were intended to be the same between the two trials. However, a dilution error resulted in the discrepancy between the two trials. Nonetheless, the results are still relevant since five of the dosages still overlap.

Following the exposure, the supernatant was collected via centrifugation and stored at -80°C for further analysis. The RAW cells were then washed out with PBS and then cultured in serum free medium (DMEM and 1% penicillin and streptomycin) for 24 hours. The serum free supernatant was then collected again and stored at -80°C for later use. The RAW cells were then washed with PBS and lyses buffer in order to collect the protein.

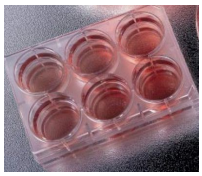
BAEC (bovine aortic endothelial cells) cells were then cultured in DMEM with 20% FBS and 1% antibiotics and transferred in a six well plate. The BAEC cells were then exposed to the serum free supernatant from the RAW cells and exposed for 24 hours. Again, the supernatant was collected and stored at -80°C and the cells were cultured in serum free medium for another 24 hours. The supernatant was then collected and the cells lysed and stored.

Figure 1



Modified from: http://quest.nasa.gov/

RAW cells were cultured in DMEM



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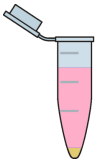
RAW cells were transferred to six well plates until 100% confluence

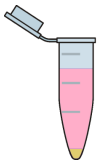
16 hour exposure to $PM_{2.5}$ and/or Ni



Modified from: http://www.clker.com/

Cells were centrifuged and the supernatant was removed.

24 hour culture in serum free medium



Cells were centrifuged to separate supernatant and cells.

Supernatant stored as stimulus for BAEC cells. Supernatent was also used to run Boyden Chamber tests.

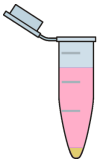
The cells were lysed and used to run Western blots for NFκB related pathways.

Figure 2

Modified from: http://www.jimbeltzer.com/

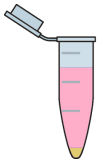
BAEC cells were cultured until 100% confluence in DMEM

16 hour exposure RAW stimulant supernatant



Cells were centrifuged and the supernatant was removed.

24 hour culture in serum free medium



Cells were centrifuged to separate supernatant and cells.

Supernatant stored to run Boyden Chamber tests.

The cells were lysed and used to run Western blots for NFκB related pathways.

Boyden Chambers

Cell migration was assayed with Boyden chambers [5.0 μm pore size, polycarbonate membrane (Neuro Probe)]. For migration assay, RAW cells were detached with a scraper and resuspended in serum free medium. After cell counting, 500 cells in serum free media (50 μL of $1x10^7$ cells/mL) were added to the upper chamber. In the lower chamber, 25 μL of the different trials with varying pollutant concentrations (chemoattractant) along with a positive (C5a) and negative (serum free medium) control were added. The transwells were incubated for one hour at 37 °C. After incubation, cells inside the inserts were removed and cells on the underside of the insert were fixed with methanol and stained with toluidine blue stain (Sigma). Transmigrated cells were counted with a light microscope, using four random regions of the membrane.

Western Blots

RAW and BAEC cells were homogenized in lysis buffer (Thermo Scientific, Rockford, IL) with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The same amounts of protein (10 ug) were separated with SDS-PAGE in 10% polyacrylamide gel and then transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were immunoblotted with primary antibodies against β-actin (1:5000), pkc-delta (1:5000), pkc-beta, (1:5000), pkc-epislon (1:5000), NFκb (1:5000), and eNOS (1:5000) followed by treatment with HRP-conjugated anti-rabbit IgG or anti-mouse IgG at a 1:5000 dilution. The membranes were detected with enhanced chemiluminescence (Super Signal West Pico; Thermo Scientific), followed by exposure to X-ray film. The protein bands on the X-ray film were scanned, and band density was calculated by ImageJ software.

Statistical analysis

Data are expressed as mean ± SE unless otherwise indicated. The results of the experiments were analyzed by unpaired t tests. In all cases, a p value of <0.05 was considered as statistically significant.

RESULTS

Chemotaxis of Macrophages Induced by the Air Pollution of $PM_{2.5}$ and/or Ni Combinations

In order to determine the positive loop of machrophage chemotaxis induced by air contaminant combinations including $PM_{2.5}$ and Ni, we used RAW 264.7 as a model cell. Chemoattractants released from the ambient air pollution exposed RAW cells were subject to Boyden Chamber experiment. (Figure 1). As shown in Figure 3, the serum free medium from RAW cells treated with either $PM_{2.5}$, Ni, or a combination of both has a significant effect on the migration of test cell RAW 264.7. For the $PM_{2.5}$ only trial, significant statistical change started at concentrations of 6.25 ug/ml. This cell movement peaks at 25 ug/ml and starts to decrease at higher concentrations of 50 ug/ml and 100 ug/ml. For the Ni only trial, significant statistical change started at concentrations of 10^{-10} M and increases up to 10^{-8} M. For the $PM_{2.5}$ + Ni trial, statistical significant change started at Ni concentrations of 10^{-7} M. The decreasing cell migration detected after 25 ug/ml may be attributed to the increased cell death due to the high concentrations of contaminants. The same trend can be seen in the Ni only trial after concentrations of 10^{-8} M and for the $PM_{2.5}$ + Ni only trial after 10^{-7} M.

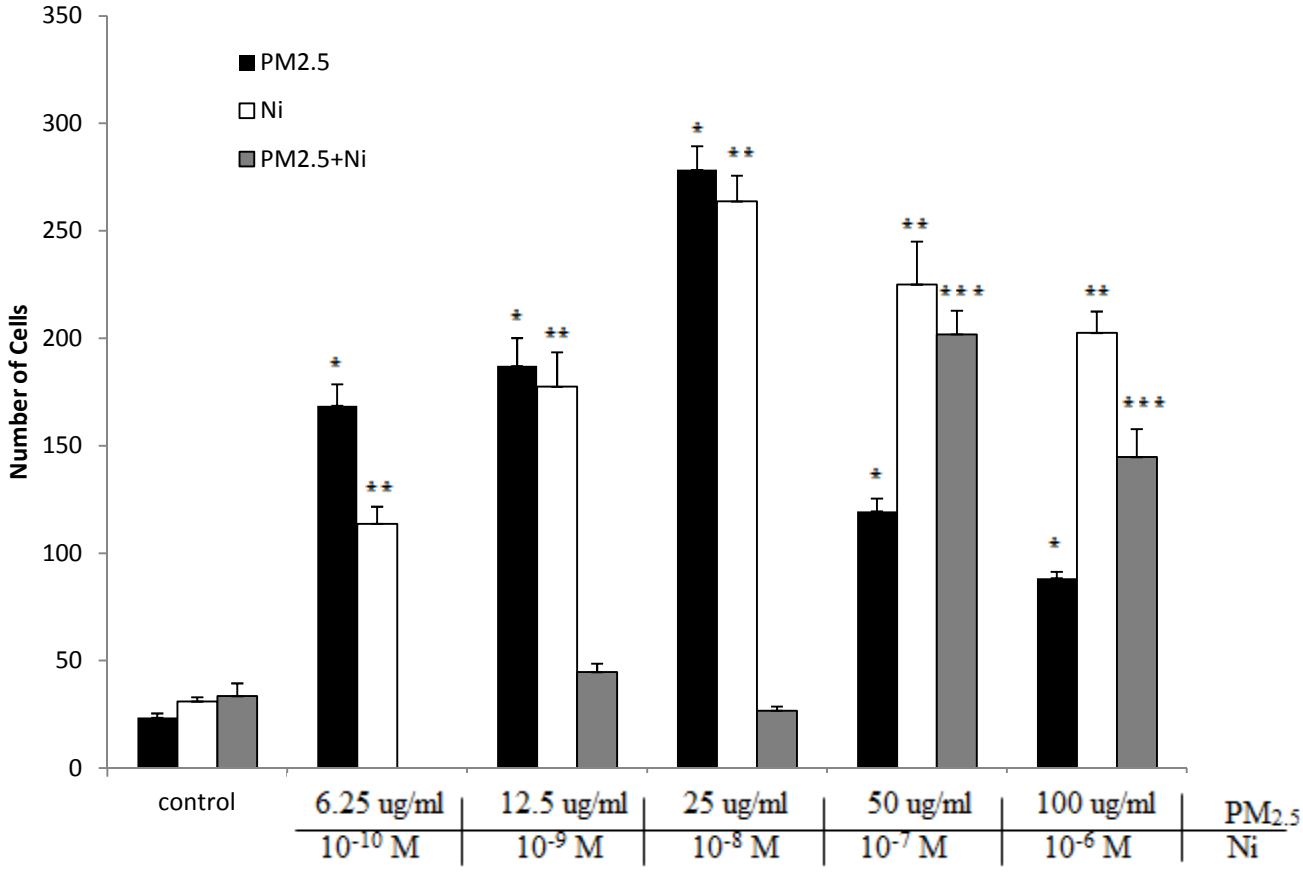


Fig 3) Determination of Cell Migrations of RAW with air pollutants of $PM_{2.5}$, Ni, $PM_{2.5}$ (25 ug/ml) + Ni
Three trials with either $PM_{2.5}$, Ni, or $PM_{2.5}$ +Ni as the stimulant for RAW cells were used in a Boyden Chamber assay to test the migration of the motile cell as a result of increased chemoattractant production. (N=6)

* = $p<0.05$ when compared to the control for $PM_{2.5}$
** = $p<0.05$ when compared to the control for Ni
*** = $p<0.05$ when compared to the control for $PM_{2.5}$ + Ni

To further understand the involvement of RAW derived chemoattractants by air pollution on endothelium cells which could trigger the cycle of RAW migrating into the vessel lining, the chemoattractant derived from the air pollutant treated RAW was applied to BAEC cells. The supernatant of BAEC cells was then used as a chemotactic factor for test RAW cells in a Boyden Chamber experiment. (Figure 2) Cell movement of RAW increased drastically even at low stimuli concentrations when compared to the control. For the $PM_{2.5}$ only trial, significant statistical change started at concentrations of 12.5 ug/ml. For the Ni only trial, significant statistical change started at concentrations of 10^{-10} M. For the $PM_{2.5}$ + Ni trial, stastical significant change started at Ni concentrations of 10^{-8} M. However, these trials did not peak at medium concentrations. This can be explained by the process in which these cells were exposed to the stimulant. Since the BAEC cell stimuli was the supernatant from the RAW trials, the effective concentrations were lower. When comparing the Ni and $PM_{2.5}$ + Ni trials, results were more aligned with my hypothesis in which the combination of $PM_{2.5}$ + Ni elicited a higher response than either stimuli by themselves.

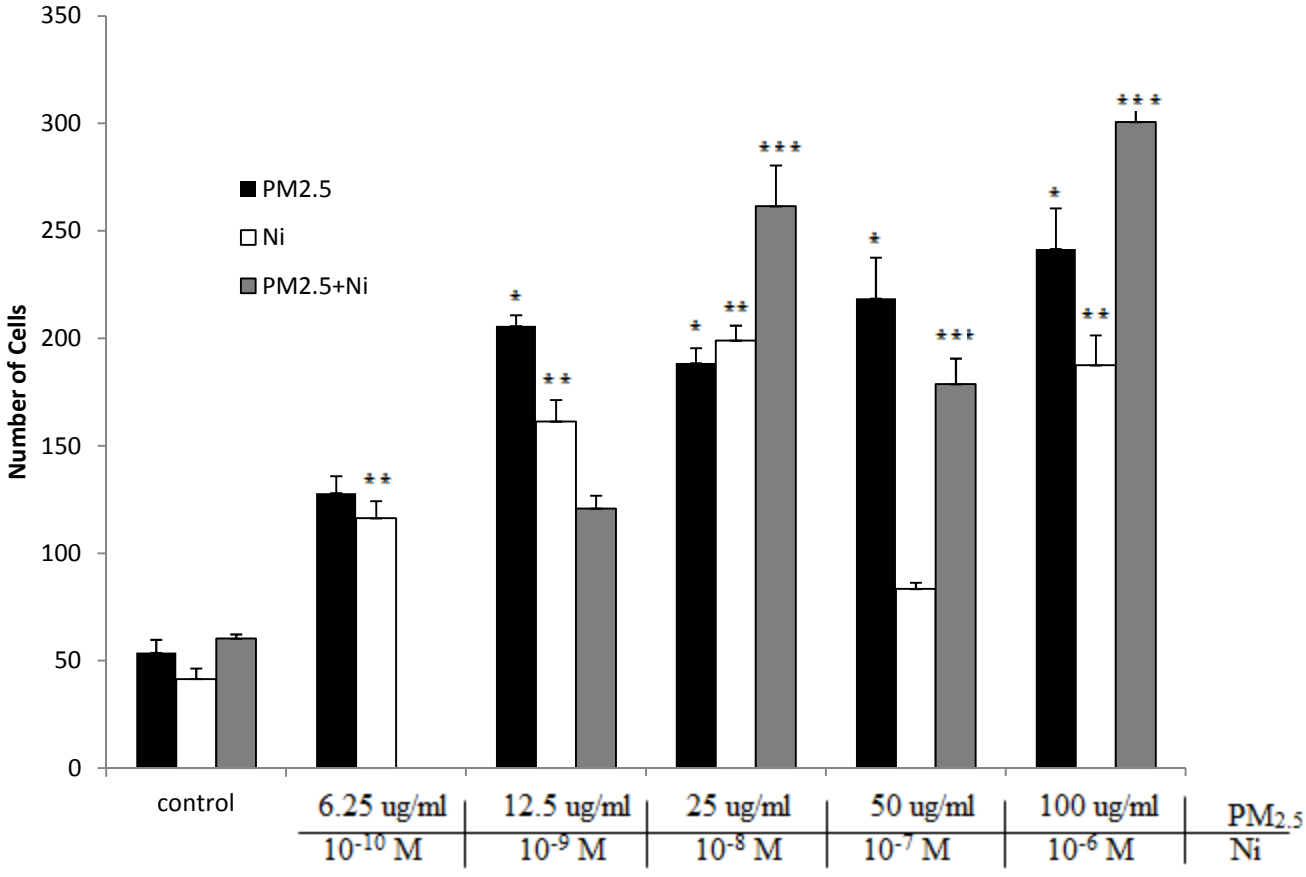


Figure 4) Determinant of Cell Migrations of BAEC with air pollutants of PM2.5, Ni, PM2.5 (25 ug/ml) + Ni
Three trials with either $PM_{2.5}$, Ni, or $PM_{2.5}$ +Ni as the stimulant for BAEC cells were used in a Boyden Chamber assay to test the change of movement in the motile cell as a result of increased chemoattractant production. (N=6)

* = $p<0.05$ when compared to the control
** = $p<0.05$ when compared to the control for Ni
*** = $p<0.05$ when compared to the control fr $PM_{2.5}$ + Ni

Activation of Cell Signaling Pathways

Following exposure to $PM_{2.5}$, there was a significant change in the NFκb expression. Figure 5a shows that the fold change increased from pollutant exposures of concentration as low as 6.25 ug/mL. There was a steady increase 50 ug/ml. While it still maintained a high level of expression, the fold changes decreased slightly from concentrations of 50 ug/ml to concentrations of 100 ug/ml. As seen in Figure 5b, NFκb expression increased greatly with increased environmental stimuli. A combined exposure to $PM_{2.5}$ and Ni resulted in great fold changes starting from the lowest concentration of 10^{-9}M and peaked at 10^{-7} M. There was a dramatic drop in NFκb expression at 10^{-6} M which can be accounted for by the increase in cell death.

Figure 5a)

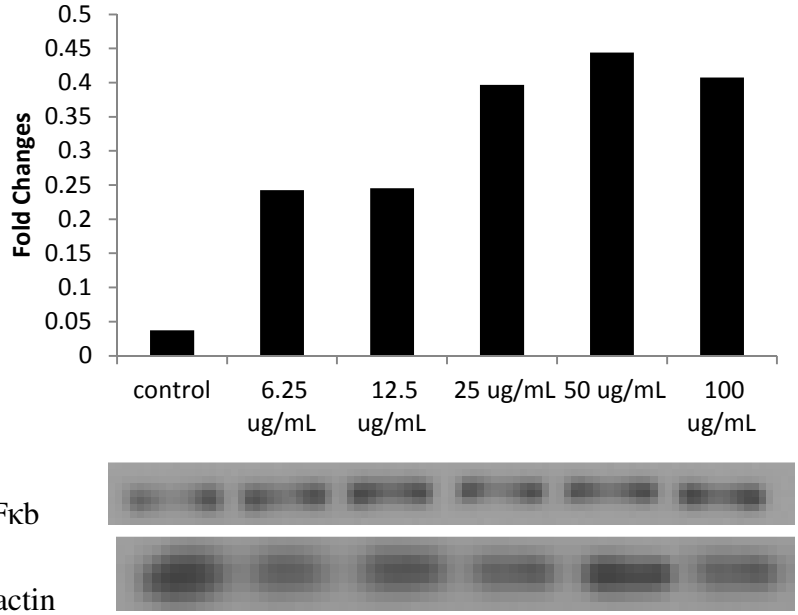


Figure 5b)

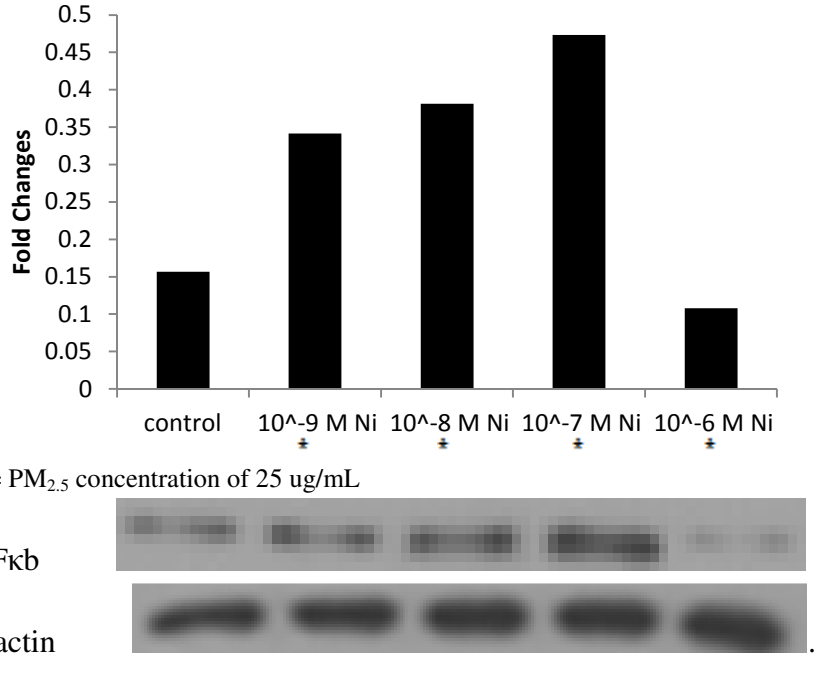
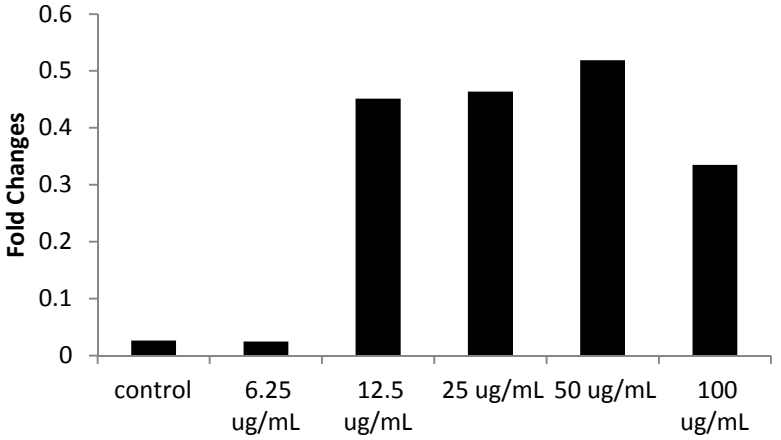


Figure 5) NFκb expression is changed by exposure of $PM_{2.5}$ and $PM_{2.5}$ + Ni for RAW cells

(a) NFκb expression was measured in RAW cells exposed to $PM_{2.5}$ from 6.25 ug/ml to 100 ug/ml. (b) NFκb expression was measured in RAW cells exposed to 25 ug/ml of $PM_{2.5}$ + Ni from 10^{-9} M to 10^{-6} M

As seen in Figure 6a, pkc-delta expression increased significantly with $PM_{2.5}$ exposure. The fold changes increased dramatically at 12.5 ug/ml and increased up to 50 ug/ml. The fold changes decreased at 100 ug/mL which could be explained with increased cell death at the higher concentration. The results from the $PM_{2.5}$ + Ni exposure shown in Figure 6b did not follow the hypothesis very well. The results were scattered with inconsistent increases and decreases in fold changes. These results could be better verified with increased trials

Figure 6a)



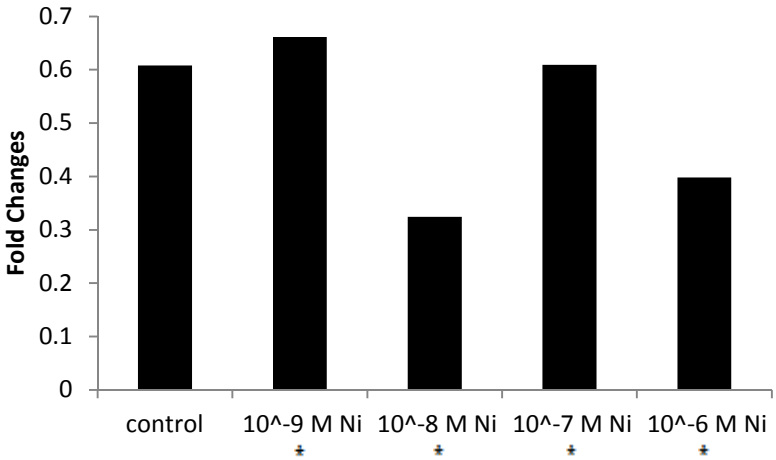
pkc-delta

β-actin

Figure 6) pkc-delta expression is changed by exposure of $PM_{2.5}$ and $PM_{2.5}$ + Ni for RAW cells

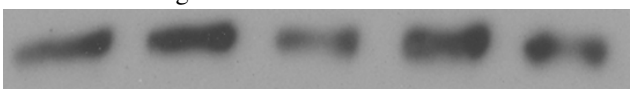
(a) pkc-delta expression was measured in RAW cells exposed to $PM_{2.5}$ from 6.25 ug/ml to 100 ug/ml. (b) pkc-delta expression was measured in RAW cells exposed to 25 ug/ml of $PM_{2.5}$ + Ni from 10^{-9} M to 10^{-6} M

Figure 6b)



* = $PM_{2.5}$ concentration of 25 ug/mL

pkc-delta



β-actin

As seen in Figure 7a, pkc-beta expression increased significantly with $PM_{2.5}$ exposure. The fold changes increased at 12.5 ug/ml and increased dramatically up to 25 ug/ml. After 25 ug/ml, the fold change began to drop. When the concentration reached 100 ug/ml, the pkc-beta reaction dropped to levels similar to the control. This decrease in fold changes can be attributed to cell death as these pollutants have reached lethal dosages. As seen in Figure 7b, the results from the $PM_{2.5}$ + Ni also showed an increased expression from as little as 10^{-9} M exposure. The fold changes then decreased with the increasing exposure. These results were inconsistent with our hypothesis and needs to be verified with more trials

Figure 7a)

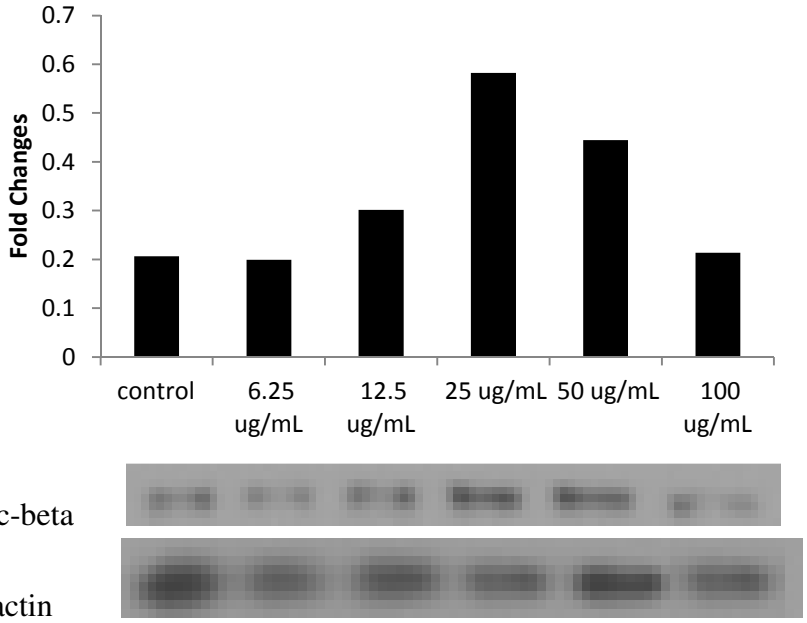
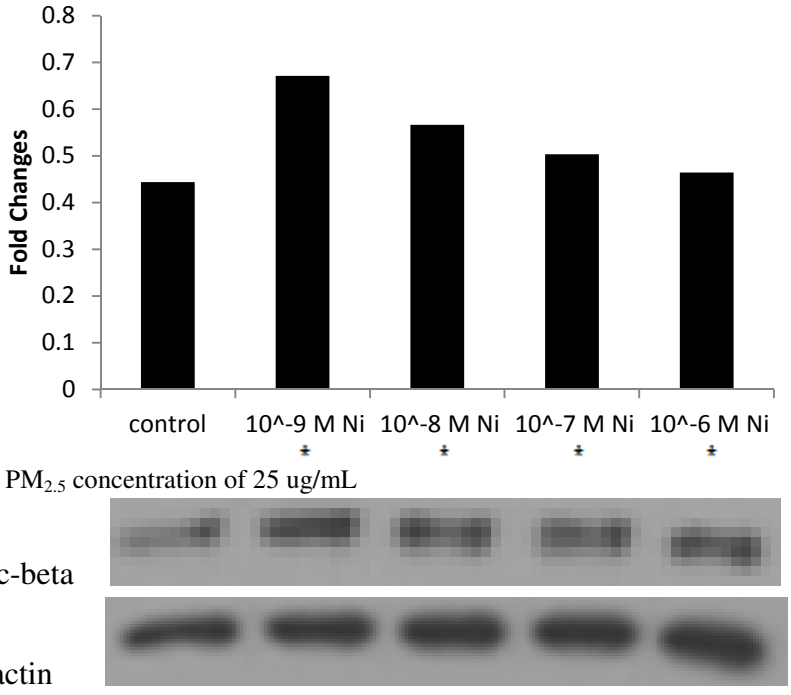


Figure 7) pkc-beta expression is changed by exposure of $PM_{2.5}$ and $PM_{2.5}$ + Ni for RAW cells
(a) pkc-beta expression was measured in RAW cells exposed to $PM_{2.5}$ from 6.25 ug/ml to 100 ug/ml. (b) pkc-beta expression was measured in RAW cells exposed to 25 ug/ml of $PM_{2.5}$ + Ni from 10^{-9} M to 10^{-6} M

Figure 7b)



* = $PM_{2.5}$ concentration of 25 ug/mL

As seen in Figure 8a, pkc-epsilon expression increased significantly with $PM_{2.5}$ exposure. The fold changes increased dramatically at 12.5 ug/ml and peaked at 25 ug/ml. The fold changes then decreased for 50 ug/ml and 100 ug/ml. The similar pattern of pkc-epsilon to pkc-beta level changes dependent on the $PM_{2.5}$ concentrations is identified and is again attributed to increased cell death at lethal dosages of pollutants. As seen in Figure 8b, RAW cells exposed to $PM_{2.5}$ + Ni also showed an increased expression from as little as 10^{-9} M exposure. The fold changes then leveled off or decreased with the increasing exposure.

Figure 8a)

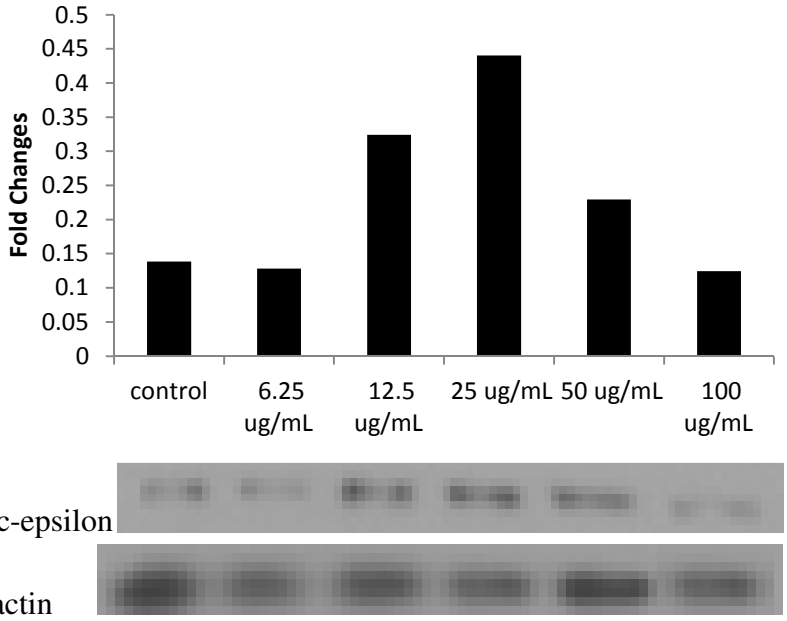
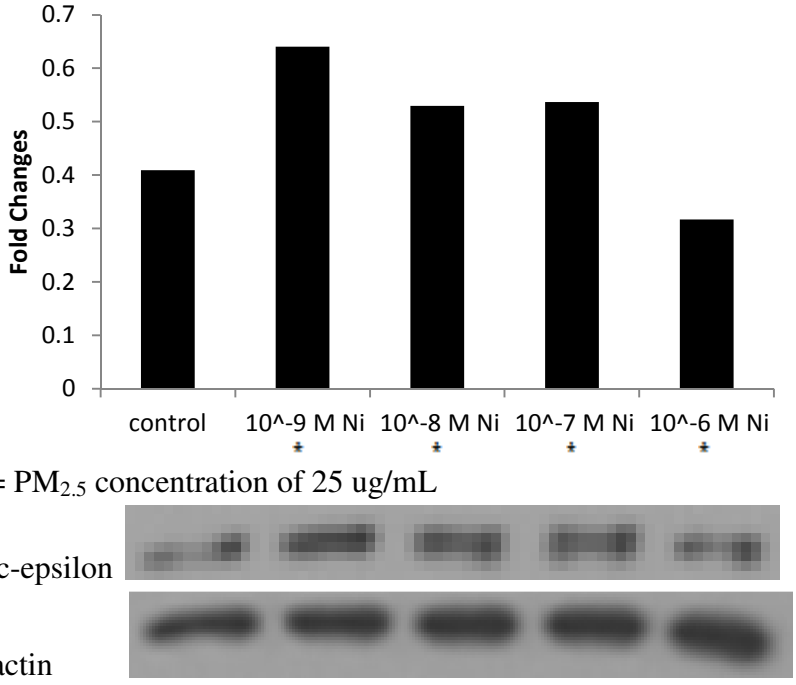


Figure 8b)



* = $PM_{2.5}$ concentration of 25 ug/mL

Figure 8) pkc-epsilon expression is changed by exposure of $PM_{2.5}$ and $PM_{2.5}$ + Ni for RAW cells

(a) pkc-epsilon expression was measured in RAW cells exposed to $PM_{2.5}$ from 6.25 ug/ml to 100 ug/ml. (b) pkc-epsilon expression was measured in RAW cells exposed to 25 ug/ml of $PM_{2.5}$ + Ni from 10^{-9} M to 10^{-6} M

Modulations of eNOS Activity

BAEC derived eNOS expressions was dramatically decreased when exposed to the supernatant pollutant $PM_{2.5}$ (Figure 9a). Even at low concentrations of $PM_{2.5}$, 12.5 ug/ml, eNOS production was decreased. With increasing concentrations, the fold changes continued to decrease and peaked at 25 ug/ml. At higher $PM_{2.5}$ concentrations, the fold changes maintained a low expression. As seen in Figure 9b, BAEC cell production of eNOS when exposed to the supernatant pollutant of $PM_{2.5}$ + Ni was dramatically decreased. Even at low concentrations of Ni, 10^{-9}M, eNOS production was decreased. This trend continued and peaked at Ni concentrations of 10^{-7} M. Ni concentrations of 10^{-7} M displayed very low production, and higher Ni concentrations maintained relatively stable with similar low fold changes

Figure 9a)

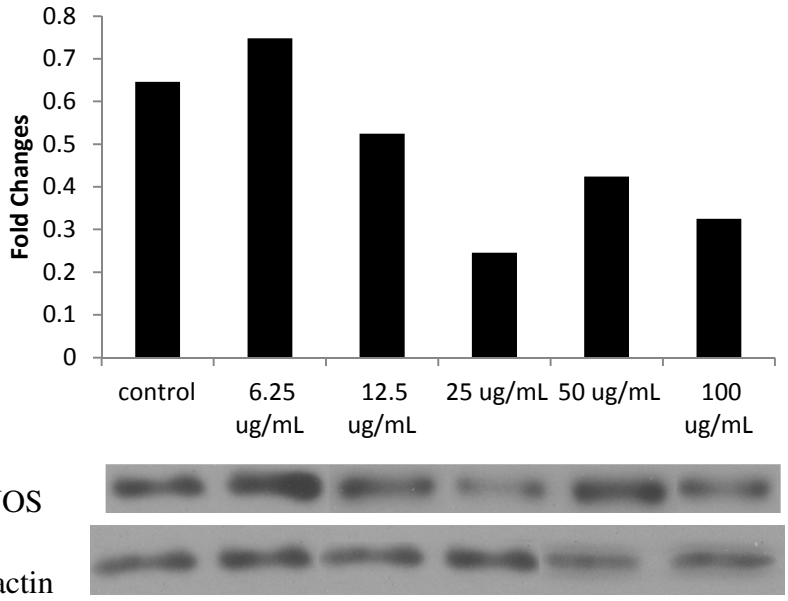


Figure 9b)

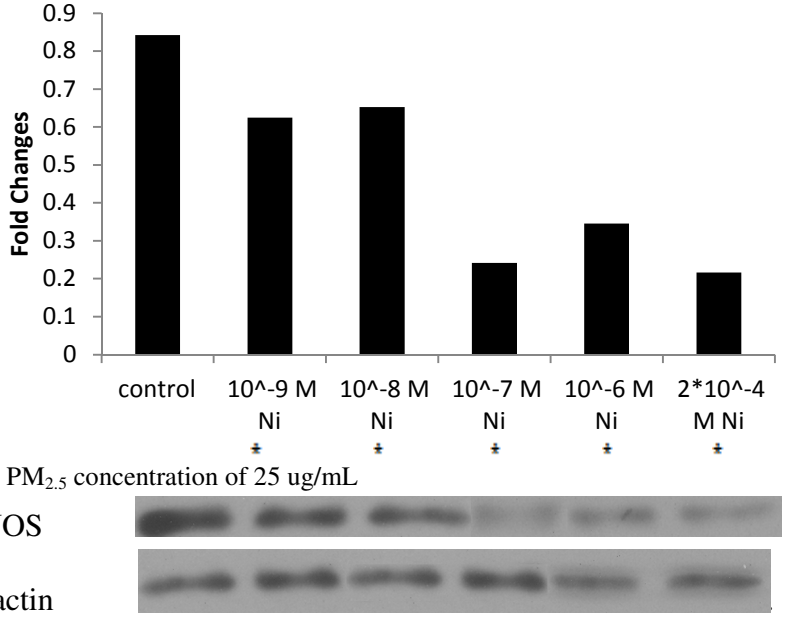


Figure 9) eNOS expression is changed by exposure of supernatant from RAW cells exposed to $PM_{2.5}$ and $PM_{2.5}$ + Ni for BAEC cells

(a) eNOS expression was measured in BAEC cells exposed to the supernatat from the RAW cells exposed to $PM_{2.5}$ from 6.25 ug/ml to 100 ug/ml. (b) eNOS expression was measured in BAEC cells exposed to the supernatant from the RAW cells exposed to 25 ug/ml of $PM_{2.5}$ + Ni from 10^{-9} M to 10^{-6} M

DISCUSSION

This study provides preliminary evidence for interactions between air pollutants and endothelial cells in terms of the eNOS functionality. First, Boyden Chamber results confirmed that the pollutant exposure could induce chemotaxis of the machrophage cells as well as activate M1 macrophage status from the RAW 264.7 cells. The graphs and figures support the hypothesis that there was indeed a trend relating the amount of chemoattractants present in the supernatant and the amount of exposure to $PM_{2.5}$ and/or nickel. This implies that the pollutant exposure elicited a response from the cells in the form of productions of cytokines and chemoattractants as a result of the $PM_{2.5}$ and/or Ni exposure.

NFκb is an important signaling pathway to study because of its implications in the cellular inflammatory response. Furthermore, NFκb is responsible for the production of many cytokines including TNF and IL-1, both of which are known to induce the M1 pro inflammatory pathway.[ix] This is especially important for its implications towards the production of eNOS. According to Western blot results, three upstream pathways, pkc-delta, beta, and epsilon, of NFκb were upregulated not only with $PM_{2.5}$ itself but were also further stimulated in combination with Ni particles. In addition, the supernatant from the activated air pollutant exposed RAW functioned as a cheomattractant for further RAW migration.

While more tests need to be run to verify conclusive data, preliminary results show that these measured responses are higher in those cells that have been exposed to $PM_{2.5}$ and/or Ni than in those of the control cells. As a result, it can be concluded that the NFκB and related pathways have been turned on in response to this environmental stimuli. This is important because these pathways are known to induce the macrophage M1 chemokine pathway that lead to inflammation as well as induce change in endothelial functions.[x]

eNOS is a well known vasodilator and the inflammation of the endothelial cells could lead to the suppression of this enzyme. This information is especially important because of eNOS's implication for atherosclerosis.[xi] Recent studies have shown a link between the suppression of eNOS and atherosclerosis which further emphasizes the need to understand our bodies' response to environmental stimuli such as $PM_{2.5}$. The expression of eNOS was measured by Western blot. From these results, while there is no definitive trend, in general, eNOS production decreases as the activated RAW from $PM_{2.5}$ and/or Ni exposures increased. This supports the hypothesis that the cell responded to these environmental stimuli through inflammation.

This study suggests that Ni exposure in addition to that already present in $PM_{2.5}$ can cause increased cellular disorders possibly through inflammatory pathways in the cells. While the concentrations of $PM_{2.5}$ and Ni were higher than that found in the environment, these were justified because the in vitro study was only 16 or 24 hours where as repeated exposure to air pollutants may last years. These results imply that exposure to $PM_{2.5}$ and Ni have an important role in cellular and metabolic disorders. Thus, an understanding of the mechanism in which the body biochemically and physiologically responds to these air contaminants may contribute to increased understanding for the prevention and treatment of different disorders such as obesity, diabetes, insulin resistance, and cardiovascular disease.[xii]

POSSIBLE FUTURE WORK

It is necessary to compare results from all three trials of $PM_{2.5}$, Ni, and $PM_{2.5}$ plus Ni. Unfortunately, many of the samples from the Ni only trial were contaminated or lost when I began running Western blots. To achieve more comprehensive results, more tests would have to be repeated to verify the trends seen in these tests. Given more time and resources, other transition metals, such as Mn, Al, and Cu, would be tested in conjunction with the $PM_{2.5}$ exposure. Since these metals are all known to be a part of the composition of $PM_{2.5}$, the similarities and differences of these individual metals would be further studied. Furthermore, the results of adding or removing Ni exposure to the cell will be directly compared. This would be achieved by having a more complete data set including all trials. This could then be expanded upon to include the other transition metals present in $PM_{2.5}$. In addition, measurements of cell necrosis and apoptosis from the exposure to pollutants will be used to better analyze and quantify the cellular responses.

Next, the varieties of cytokines released by the cells when induced by air pollutants will be screened. In particular, the expressions of chemoattractants such as IL-1, IL-6, TNF, TGF-α, and TGF-β would be most immediately investigated. These chemoattractants are important because they have major functions in inducing the macrophage M1 chemokine pathway that lead to inflammation as well as induce change in the endothelium cells. ELISA (Enzyme-Linked ImmunoSorbant Assay) could also be used to specify which key cytokines were involved in the activated macrophages and the RAW recruitment. By expanding upon the different components of $PM_{2.5}$ and the cytokines produced, we could further understand the implications of $PM_{2.5}$ exposure as well as the cellular response to these environmental pollutants.

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